
Nucleotide sequences of mouse genomic loci including a gene or pseudogene for U6 (4.8S) nuclear RNA

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ABSTRACT

We have isolated four clones which hybridize with U6(4.8S) nuclear RNA, a mammalian small nuclear RNA(snRNA), from DNA of BALB/c mouse liver. Their restriction maps are totally different from each other, indicating that they derived from different loci in the mouse genome. The nucleotide sequences around the hybridizing region in the three clones have been determined. One clone gives a gene that is co-linear with the U6 RNA. There is a sequence TATAAAT beginning 31 nucleotides upstream of the gene, which may suggest that the U6 RNA is transcribed by RNA polymerase II. The other two clones contain a pseudogene for the U6 RNA which has 7 or 9 nucleotide changes from the RNA. The pseudogenes are surrounded by radically different sequences from those surrounding the gene, and they are closely linked to a pseudogene for another snRNA, 4.5S-I RNA, or a part of highly repetitive and interspersed sequence B1.

INTRODUCTION

Eukaryotic nuclei contain a group of small stable RNAs which are called as small nuclear RNA or snRNA(1, 2). Their functions have long been obscure. However, Lerner and Steitz (3) and Lerner et al.(4) recently suggested that several mammalian snRNAs such as U1 are involved in RNA splicing. More recently, an evidence has been reported indicating that U1 RNA is actually involved in splicing(5). As one of the approaches to elucidate the functions of snRNAs, we have undertaken isolation and characterization of mammalian genes for these RNAs. In this article, we report on cloning and sequence determination of mouse loci which have homology with U6(4.8S) nuclear RNA. It is one of the major snRNAs in mammals(3,4,6-8). Although the function of the U6 RNA is not established, it might be involved in splicing since it is complexed with the

same proteins as is U1 RNA(3,4,7) and since it has a sequence which is complementary to exon-intron junctions of certain mRNA precursors(6). The nucleotide sequence of U6 RNA has been reported for mouse(6) and rat Novikoff hepatoma(8). The sequence appears to be most strictly conserved in mammals and in chicken(F. Harada, personal commun.).

MATERIALS AND METHODS

Preparation of snRNAs

Nuclei were prepared from 8 week male Wistar rats as described(9). RNA was extracted from the nuclei by SDS-hot phenol method(10), which was fractionated by sedimentation through 20-5%(W/V) sucrose gradient in 10mM Tris.HCl(pH7.5)/0.1M NaCl/1mM EDTA/0.1% SDS. Fractions smaller than 10S were pooled and precipitated with ethanol(crude snRNA fraction). The RNA was subjected to electrophoresis in 10% acrylamide gel in 0.3xTEB(11) (1xTEB=89mM Tris/89mM boric acid/2.5mM Na-EDTA). SnRNAs were detected under ultraviolet ray on a fluorescent cellulose sheet or after staining with toluidine blue. Each snRNA band was excised and the RNA was extracted by immersion of the ground gel in 0.5M ammonium acetate/10mM magnesium acetate/0.1% SDS/0.1mM EDTA with shaking for several hours at room temperature. RNA was precipitated with ethanol and further purified by electrophoresis in 20% acrylamide gel in 0.5xTEB. Either the purified snRNA or crude snRNA fraction was partially cleaved with heating in formamide(12) and labeled with γ -³²P-ATP and polynucleotide kinase for the probe used in hybridization for cloning and analysis.

Isolation of recombinant phage clones

High molecular weight DNA was extracted from male BALB/c mouse liver principally as described(13). The DNA was partially digested with EcoRI into fragments of 10 kilobases(kb) on the average and subjected to sedimentation in sucrose gradient to remove fragments smaller than 5-6kb. The arms of the phage vector λ gtWES. λ B(14) were prepared as described(15), and ligated with mouse EcoRI fragments by T4 DNA ligase. The recombinant DNA was packaged in vitro according to F. Blattner's protocol to yield 10^6 - 10^7 phages/ml packaging mixture on DP50supF.

Screening of phage clones were done in three steps with the plaque hybridization method(16). About 200,000 plaques($1-2 \times 10^4$ /plate) were screened with ^{32}P -labeled snRNA mixture as the 1st step to give about 20 positive spots. After 2nd screening for purification, positive clones were hybridized with purified U1, U2, U5, U6 or 4.5S RNA. Four independent clones hybridized strongly with the U6 RNA. About 10 other clones hybridized other snRNAs, which are not mentioned in this report.

Subcloning

Phage clones 52, 34 and 55 were digested with BamHI/EcoRI, Hind3/EcoRI or BamHI/EcoRI, respectively, and ligated with plasmid pBR322(17) digested with BamHI/EcoRI or Hind3/EcoRI. E.coli χ 1776 was treated with MnCl_2 - CaCl_2 for transformation as described(18) with a modification. Ampicilin-resistant transformants were screened by colony hybridization with ^{32}P -U6 RNA. Our colony hybridization procedure deleted all the suction steps and treatment with chloroform for DNA fixation from that described(19). Plasmids were prepared by 2 cycles of equilibrium centrifugation in CsCl-ethidium bromide of the cleared lysate(20).

Analysis of clones

Electrophoresis of restriction fragments in agarose gel was done in a horizontal apparatus(21). Southern blot(22) was done using Schleicher-Shuell BA-85 nitocellulose sheet and $10\times$ SSC. Hybridization was done in $4\times\text{SSC}/10\text{mM}$ K-phosphate(pH 7.0) at 65°C overnight. Electrophoresis of DNA fragments in acrylamide gel was done as described(23,24). For isolation of DNA fragments separated in agarose gel, DNA was eluted electrophoretically(21). DNA in acrylamide gel was eluted by immersion of crushed or ground gel. Kinase label of DNA fragments and sequencing reaction were done according to Maxam and Gilbert(23,24).

Biohazard containment

All the handling of cells containing portions of mouse genome was done in an EK2 host vector system and in the P2 level Recombinant DNA facility in the Insitute of Biological Sciences, University of Tsukuba according to the Guidelines for Recombinant

DNA research from Ministry of Education of Japan.

RESULTS

Construction and mapping of recombinant phage clones which hybridize with the U6 RNA

The DNA of BALB/c mouse liver was partially digested with restriction endonuclease EcoRI and sedimented through sucrose gradient to give fragments of about 10kb(kilobases) on the average. The EcoRI fragments were ligated with the arms of phage vector λ gtWES. λ B(14) and the recombinant DNA was packaged in vitro. About 200,000 phage plaques were screened by hybridization with rat liver U6 RNA, and four positive clones were obtained.

The phage clones were analyzed with restriction endonucleases of 6 base-pair(bp) recognition and electrophoresis in agarose gel. The region which hybridizes with the U6 RNA in each clone was localized by Southern blot-hybridization(22). The results are shown in Fig.1. The restriction maps of the clones are totally different from each other, indicating that

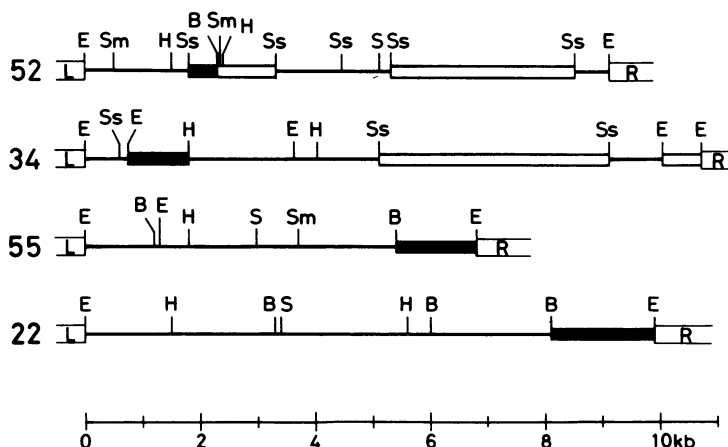


Figure 1. Maps of the sites sensitive to restriction enzymes of 6bp recognition in the inserts of the phage clones. Thick solid lines represent regions hybridizing with rat U6 RNA. Open rectangles show fragments hybridizable with the B1 sequence. L and R represent left and right arms of the vector λ gtWES, respectively. E=EcoRI, H=Hind3, B=BamHI, S=Sall, Ss=SstI and Sm=Smal.

they have originated from different loci in the mouse genome. From each of the three clones (52, 34 and 55 in Fig.1) an appropriate fragment including the hybridizing region was subcloned, and detailed restriction maps were constructed (Fig.2). The maps around the hybridizing regions are again different from each other.

A gene in clone 52

The nucleotide sequences of the hybridizing regions in the three clones have been determined according to the strategy in Fig.2 and they are shown in Fig.3. The sequences which have homology with the U6 RNA are aligned at positions 1-106 or 107 and shown in large capital letters. The clone 52 is concluded to contain a real gene for U6 RNA, although the sequence shown is different at two positions from that of mouse U6 RNA reported by Harada et al. (6). One difference is that between TTAG at position 51-54 in DNA and UUUAG in RNA. For this position, the DNA sequence is considered to be correct (RNA sequence should be UUAG) for three reasons. First, Harada et al. (6) also obtained sequence UUAG by chemical sequencing of mouse liver U6 RNA. The final sequence they reported is based on the analysis of uniformly labeled RNA from cultured cells by finger-print analysis,

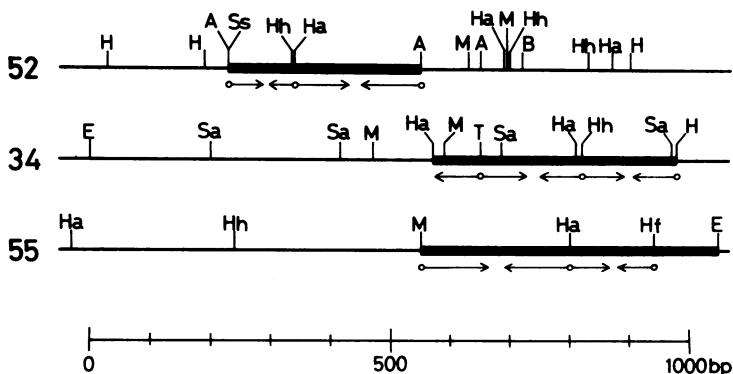
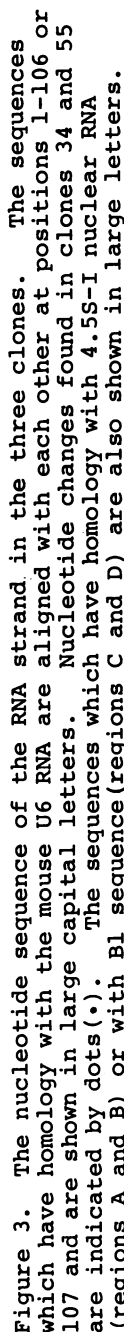


Figure 2. Fine maps of the restriction sites around the hybridizing regions in the three clones. Thick solid lines represent fragments hybridizing with the U6 RNA. Arrows below each map show the sequencing strategy. E=EcoR1, H=Hind3, B=BamH1, Ss=Sst1, Ha=Hae3, Hh=Hha1, A=Alu1, M=Msp1, T=Taq1 and Sa=Sau3A.



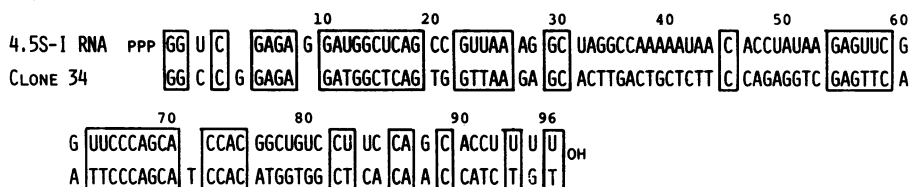
which might sometimes be difficult to interpret. Second, rat U6 RNA has also sequence UUAG at this position(8). Third, the sequences in clones 34 and 55 are also TTAG. The other difference is that between the 3'-terminal 4T in DNA and 5U in RNA. For this difference, the reason is probably that there are genes of 4T as well as 5T since clone 34 has also 4T whereas clone 55 has 5T. Rat U6 RNA has corresponding terminal heterogeneity consisting of 4U and 5U(8) in accordance with this view. For these reasons, it is likely that the sequence 1-106 in clone 52 is a gene that is co-linear with U6 RNA in BALB/c mouse liver. The gene is not split by an intervening sequence. In the flanking region 5' to the gene, there is a Hogness box sequence TATAAAT begining at position -31 which is boxed in Fig.3(see Discussion).

Pseudogenes in clones 34 and 55

The sequences in clones 34 and 55 which have homology with the U6 RNA are different at 7 or 9 positions from the RNA sequence as indicated by dots in Fig.3. These sequences can be regarded as pseudogenes(25) or non-functional genes. The reasons will be described in Discussion.

The sequences immediately adjacent to the pseudogenes are radically different from those surrounding the gene in 52 and are very rich in A. In the 5'-flanking sequences no Hogness box sequence is found. Instead, in clone 34, there are two loci (regions A and B in Fig.3) that have homology with a part of another snRNA, 4.5S-I RNA(26). The sequences around the loci are compared with that of rat 4.5S-I RNA in Fig.4a. The 4.5S-I RNA consists of 96 nucleotides(26) and its function is unknown. In clone 55, the 5'-flanking region contains an interrupted B1 sequence(region C in Fig.3, -152 to -58). The B1 sequence which was reported by Krayev et al.(27) is shown in Fig.4b in comparison with that of region C in clone 55. The reported B1 sequence is 130bp in length, highly repetitive (40,000-80,000 copies/haploid) and interspersed in the mouse genome(27). It is complementary to the most abundant class of mouse fold-back RNA and are homologous to Alu family sequences which are highly repetitive and interspersed in the human genome (28-30). Both the reported B1 sequence and that in clone 55

(a)



(b)

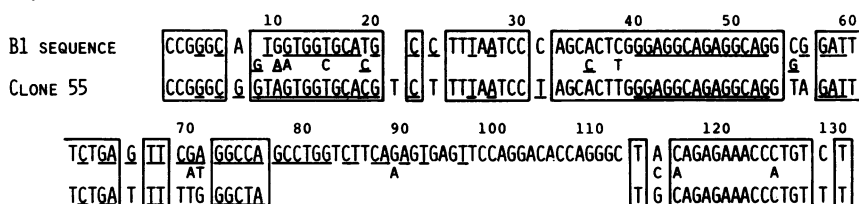


Figure 4. (a) The nucleotide sequence of rat 4.5S-I RNA (26) compared with the DNA sequence in clone 34 around regions A and B. Homologous regions are boxed. (b) The nucleotide sequence of the mouse B1 sequence (27) compared with that of region C in clone 55. Homologous regions are boxed. The top line represents the consensus nucleotides among B1-a, B1-b and B1-c and the second line represents an alternative one, if any (27). Sequences which are represented in RNA-bound 4.5S RNA in mouse (31) are underlined.

contain sequences (underlined in Fig. 4b) that are represented in RNA-bound 4.5S RNA in mouse (31). It is different from 4.5S-I nuclear RNA and was found in complexes with poly(A)-RNA both in cytoplasm and in nuclei (31,32). One of the regions represented in the RNA (GGAGGCAGAGGCAG from 40 to 53 in Fig. 4b) is closely related to a presumed viral replication origin (28). Clone 34 also contains a part of B1 sequence (from 9 to 59 of Fig. 4b), in this case in the 3'-flanking region (region D in Fig. 3).

The sequence arrangement in the three clones is summarized in Fig. 5. The polarity of the B1-related sequences, which is defined as the direction of transcription of the RNA-bound 4.5S RNA partly included in the sequences or as supposed from that of related Alu-family sequences (28,30,33), is in the same orientation with that of the pseudogenes in both clones.

Other B1-related sequences in the original phage clones were looked for by hybridization with that in clone 55 (region C,

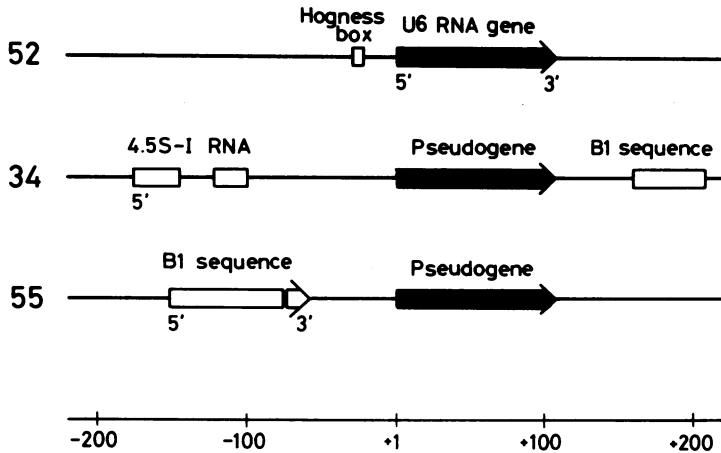


Figure 5. Summarized sequence arrangement in the three clones around the gene or pseudogene.

MspI-Taq I 96bp fragment) which had been labeled by nick-translation(34). The results are shown in Fig.1 where a fragment hybridizable with the probe is represented by an open rectangle. In clone 52, two fragments upstream of the U6 RNA gene contain a B1-related sequence. In clone 34, two more fragments besides the sequenced Eco-Hin fragment contain a B1-related sequence (both downstream of the pseudogene). In clone 55, other fragments than the Bam-Eco fragment do not contain a detectable B1-related sequence, and in clone 22 not at all. Thus at least six B1-related sequences are found in the four clones spanning 36kb in total, which supports the view that B1 sequences are highly repetitive and interspersed.

DISCUSSION

We have isolated four phage clones from mouse genome which hybridize with the U6 nuclear RNA. One of the clones(clone 52) contains a sequence that is co-linear with the mouse U6 RNA. It is very likely to be a gene that is producing a functional U6 RNA *in vivo*. Isolation of a chicken gene for U1 RNA has recently been reported(35).

The canonical sequence TATAAAA and its minor variations

called as Hogness box sequences are found around 30 nucleotides upstream of most genes transcribed by RNA polymerase II (36). Therefore finding of sequence TATAAAT beginning 31 nucleotides upstream of the gene for U6 RNA (in clone 52) may suggest that the U6 RNA is transcribed by RNA polymerase II. In the case of the chicken gene for U1 RNA (35), no Hogness box was found in spite of a supporting evidence for transcription by RNA polymerase II. It is presumed that a Hogness box sequence does not necessarily precede a gene for a snRNA even if it is transcribed by polymerase II. There is a short palindromic sequence just after the 3'-end of the gene in clone 52 indicated by the arrows in Fig.3. It might have a role in termination of transcription.

We have found sequences that have homology with, but are significantly different from, that of the U6 RNA in clones 34 and 55. The reasons for which we regard these sequences as pseudogenes or non-functional genes are two-fold. First, the sequence of U6 RNA appears to be most strictly conserved in mammals on the basis of finger-print patterns (F. Harada, personal commun.), a situation similar to that found for U1 RNA (4,37). Second, the sequences surrounding these U6 RNA-related sequences are quite different from those surrounding the real gene in clone 52. They are rich in A and appear largely to be composed of simple repeating units. Thus the surrounding sequences are not likely to support correct initiation or termination of transcription of the "gene". The large difference in the surrounding sequences among the three clones do not support the view that the U6 RNA-related sequences in clones 34 and 55 code for a minor component of U6 RNA. For these reasons, the two clones out of three sequenced are likely to include a pseudogene. The fourth clone (clone 22) also seems to include a pseudogene because of somewhat weaker hybridization with the U6 RNA. Denison et al. (38) have recently reported that pseudogenes for snRNAs are quite prevalent in the human genome. Our results provide a further evidence indicating that pseudogenes for an snRNA are prevalent in mammalian genomes.

The fact that the four clones isolated in this report have different restriction maps indicate that they derived from

different loci in the mouse genome. The result is consistent with the view that genes or pseudogenes for U6 RNA are repetitive and interspersed as has been suggested for some snRNA genes (39-41). This view is further supported by the analysis of total mouse DNA by digestion with EcoRI and Southern blot-hybridization with the U6 RNA. Hybridization was detected with the fragments from 2 to 24kb continuously with two barely detectable bands at 8 and 14kb (data not shown). Tens or more of regions hybridizable with the U6 RNA are supposed to exist in the mouse genome, most of which may be pseudogenes.

As for sequences related to other snRNAs in each of the four clones, those which are related to U1, U2 or U5(5S) RNA were not detected by hybridization of 3'-end labeled snRNA to the phage DNA followed by elution and analysis of RNA in acrylamide gel (38,42). Presence or absence of sequences related to less abundant snRNAs were not tested except for the sequenced regions.

In the regions flanking the pseudogenes, interesting sequences were found: an interrupted and divergent gene for 4.5S-I RNA in clone 34 (5'-side), an interrupted or truncated B1 sequence (5'-side in clone 55 or 3'-side in clone 34; Figs. 3-5). Alu family sequences which are related to the B1 sequence and highly repetitive in the human genome have been found preceding pseudogenes for snRNA (38). Since B1 sequences are highly abundant, the interrupted or truncated B1 sequence in clone 55 or 34 might be related to the abundance of pseudogenes for U6 RNA. This possibility is supported by the fact

that the pseudogenes are closely linked to B1-related sequences and the probably functional gene in clone 52 is not. Alternatively, since Alu or B1 sequence has been suggested to be a transcription unit by RNA polymerase III (28,30,33), the B1-related sequence in clone 55 or 34 might have been functional as such a unit. The interrupted and divergent gene (pseudogene) for 4.5S-I RNA in clone 34 is located to the 5' of, and in the same orientation with, the U6 RNA pseudogene. 4.5S-I RNA is supposed to be transcribed by RNA polymerase III since it has 5'-terminal pppG and a sequence GUUCGGUCC (26) that is presumably a major recognition site for correct initiation by polymerase III (43). The sequences related to 4.5S-I RNA in clone 34 might

be or might have been functional as a transcription unit by polymerase III. Thus clones 34 and 55 may provide a further support for the view that a transcription unit by RNA polymerase III often precedes a transcription unit by polymerase II(38).

There are two notable direct repeats in clone 52 surrounding the gene: CACCCTAAC begining at positions -65 and +129, and TTTCTAT at -124 and +163(both underlined in Fig.3). In clones 34 and 55, 4bp repeat CCCT included in the repeat CACCCTAAC is found in the corresponding regions(underlined). CCCT is the only sequence found in common to the three clones in the corresponding regions. These results may suggest a functional role for the related repeats CACCCTAAC and CCCT. They might be related to a mechanism similar to transposition(44).

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